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C-CAM is a tumor suppressor that is lost early in the progression of prostate cancer. The mechanism of C-CAM down-regulation in tumorigenesis is not clear. We propose to unravel the mechanism that down-regulates C-CAM gene expression in prostate carcinogenesis. Identification of early genetic changes in tumorigenesis might yield targets for early prevention and detection. We have identified transcriptional factors, including AP-2 and androgen receptor, that are involved in the regulation of C-CAM gene expression. In addition, we have developed an in vivo functional screening method to identify new transcriptional factors that regulate C-CAM gene expression during prostate carcinogenesis. Results from this study will allow us to design new therapy strategies to alter tumor progression or to implement early detection and prevention strategies.

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Shu-Hwa Lin 8/30/99

PI - Signature Date

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(A) Introduction

C-CAM is a tumor suppressor that is lost early in the progression of prostate cancer. The mechanism of C-CAM down-regulation in tumorigenesis is not clear. There is no evidence that human C-CAM gene, which is located on chromosome 19, is deleted in prostate cancer patients. Furthermore, decreased expression, but not mutation, of C-CAM is detected at early stages of prostate carcinogenesis. We propose to unravel the mechanism that down-regulates C-CAM gene expression in prostate carcinogenesis. Identification of early genetic changes in tumorigenesis might yield targets for early prevention and detection. We will identify factors that alter C-CAM expression in malignant transformation. Specifically, we plan to: (1) Examine whether methylation of C-CAM gene is a mechanism for C-CAM down-regulation in prostatic carcinogenesis; and (2) Characterize transcription factors that regulate C-CAM expression in prostatic carcinogenesis.

(B) Progress Report

We proposed to investigate the tumor-specific regulation of C-CAM cell adhesion molecule in prostate carcinogenesis. The proposed work was divided into four Tasks to be carried out in parallel.

Task 1. Examine whether cytidine methylation of regulatory sequences in C-CAM gene occurs in human prostatic carcinogenesis (months 1-18)

Task 2. Examine the involvement of AP-2 in C-CAM gene expression in carcinogenesis (months 1-12)

Task 3. Search for the transcriptional activators/co-activators involved in C-CAM expression (months 1-30)

Task 4. Search for transcriptional repressors (months 1-24).

In this section, we will describe our progress in this study. Since Task 3 will require the entire project period (months 1-30) to complete, we immediately started on this Task and obtained a substantial amount of interesting results. This part of work will be described first.

A reviewer for our proposed work suggested that we pursue regulation of C-CAM gene expression by steroid hormones. We agree with

this reviewer that steroid hormones are important regulators of prostate growth and differentiation and, therefore, we have included androgen regulation in our study of transcriptional regulation of C-CAM gene. With the assistance of Dr. Guido Jenster, an expert in the field of gene regulation by steroid hormones and a collaborator in the proposed study, we have made significant progress in this effort and a manuscript has been prepared for publication. We will briefly describe this part of the work, which is described in more detail in the attached manuscript .

Task 2 is to examine the involvement of AP-2 in C-CAM gene expression in carcinogenesis. We have obtained an expression plasmid containing AP-2 cDNA from Dr. Michael Tainsky of Karmanos Cancer Center, Detroit, Michigan and have started this part of study. We will describe some results obtained so far.

B.1. Studies performed under Task 3--Search for the transcriptional activators/co-activators involved in C-CAM expression

B.1.1 Rationale

We hypothesized that decrease in C-CAM transcription during tumorigenesis may be due to loss of activator(s) or coactivator(s). A transcription activator which is involved in the regulation of C-CAM gene expression during tumorigenesis should have the following properties: (1) It should exhibit different levels of expression between normal and cancer cells. (2) It should be able to activate C-CAM expression in C-CAM negative cells. (3) Restoration of its expression should suppress tumorigenicity of prostate cancer cells. We will identify the potential tumor-specific transcription factors based on these criteria.

B.1.2. Experimental plan

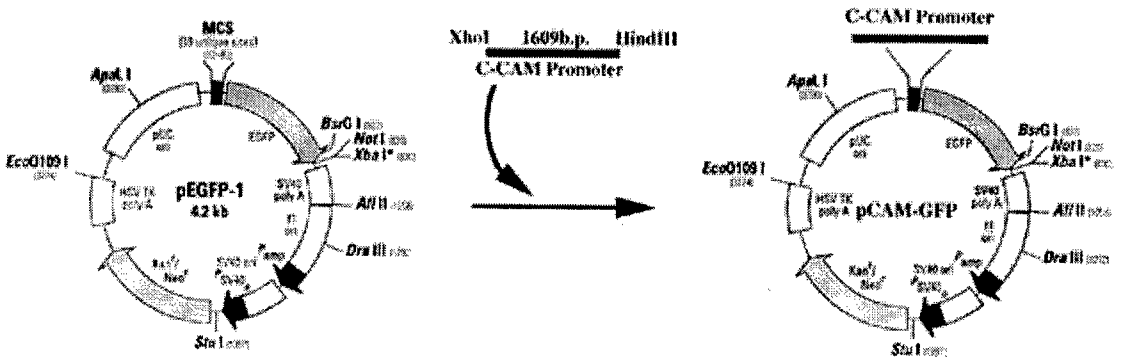
We plan to functionally identify these factors in the context of natural C-CAM promoter. In brief, the C-CAM promoter will be constructed in front of a green fluorescence protein (GFP) reporter gene. Activation of C-CAM promoter by transcription factors will stimulate the expression of GFP. Cells with elevated level of GFP can be selected by fluorescence-activated cell sorting (FACS) analysis. Plasmids in GFP-positive cells will be isolated for further analysis.

B.1.3. Results

B.1.3.1. Construction of C-CAM promoter into pEGFP-1 promoter-reporter vector to generate plasmid pCAM-GFP (as proposed)

The 1609 bp C-CAM promoter was isolated from plasmid pGL-1609 by XhoI and HindIII restriction-enzyme digestion. This 1609 bp promoter sequence was then cloned in front of green fluorescence protein gene in the reporter plasmid, pEGFP-1 (Clontech, CA). The map of the resulting plasmid, pCAM-GFP, is shown in Fig. 1.

Fig. 1. Construction of GFP reporter plasmid containing C-CAM1 promoter.



B.1.3.2. Transfect pCAM-GFP into PC3 cells and select for transfectants by G418 to establish PC-CAM-GFP cells (as proposed)

The plasmid pCAM-GFP was transfected into PC3 cells with the aid of DOTAP (Behringer Menheim Biochemical) and the cells that contained the pCAM-GFP plasmid were selected by G418. Several clones (PC-CAM-GFP) were selected and expanded for further study.

B.1.3.3. FACS and western blot analysis of PC-CAM-GFP cells (as proposed)

The C-CAM promoter activity in PC-3 cells was measured by FACS and western blot analysis. Six PC-3 cell lines (PC-CAM-GFP-1, PC-CAM-GFP-2, PC-CAM-GFP-3, PC-CAM-GFP-4, PC-CAM-GFP-5 and PC-CAM-GFP-6) were tested and the parental PC-3 cells were used as a control. Both FACS analysis and western immunoblot using polyclonal antibody against GFP were employed to measure the amount of GFP protein produced. The PC-3 control cells did not have GFP expression, while transfection of PC-3 cells with the PC-CAM-GFP construct produced 10- to 100-fold increases in fluorescence intensities, suggesting that GFP was produced from the pCAM-GFP construct (data not shown). Western immunoblot analysis showed that significant amounts of GFP protein were detected in the PC-CAM-GFP

cell lines (data not shown). This observation suggests that the increases in fluorescence intensities in PC-CAM-GFP cells indeed resulted from the production of GFP rather than from non-specific auto-fluorescence of the transfected cells.

B.1.3.4. Interpretation of PC-CAM-GFP FACS analysis results

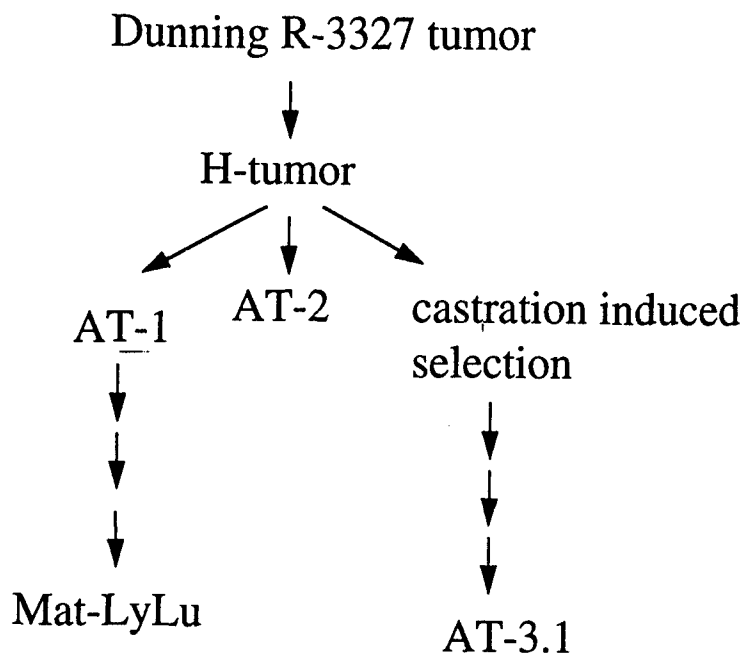
Although C-CAM is not expressed in PC-3 cells, we have observed that the 1609 bp C-CAM promoter was able to activate GFP expression in PC-3 cells. This observation suggests that either the 1609 bp C-CAM promoter region does not contain the tumor-regulated element or this 1609 bp promoter fragment is not sufficient to elicit the tumor-specific regulatory event.

B.1.3.5. Rationale for selecting Mat-LyLu rat prostate cancer cell line for further study (modification based on the negative results obtained)

Based on the result shown above, it is obvious that a longer promoter would be needed to study the tumor-specific transcriptional regulatory event. Alternatively, the study can be performed in a tumor cell line in which the level of C-CAM expression was down-regulated and modulation of C-CAM expression can be easily detected. The latter approach has the advantage of not being limited by the size of promoter obtained and also has the advantage of studying C-CAM regulation in its natural environment.

We found that Dunning rat prostate cancer cell lines have the properties that is suitable for our purpose. The Dunning 3327 prostate cell line was isolated from a rat prostate tumor by Dunning (1) from a 22-month-old inbred Copenhagen male rat. Following serial *in vivo* passage of the original R3327 tumor, sublines with different biological characteristics were obtained and characterized (Fig. 2) (2). These cell lines represent tumors ranging from relatively benign, slowly growing, differentiated, androgen-sensitive tumors to rapidly growing, anaplastic, hormone-insensitive malignant tumors. If the level of C-CAM protein expression in this series of cell lines showed distinct tumor-specific down-regulation, these cell lines will be suitable for studying tumor-specific regulation of C-CAM expression. Therefore, we first characterized C-CAM expression in this series of Dunning cell lines.

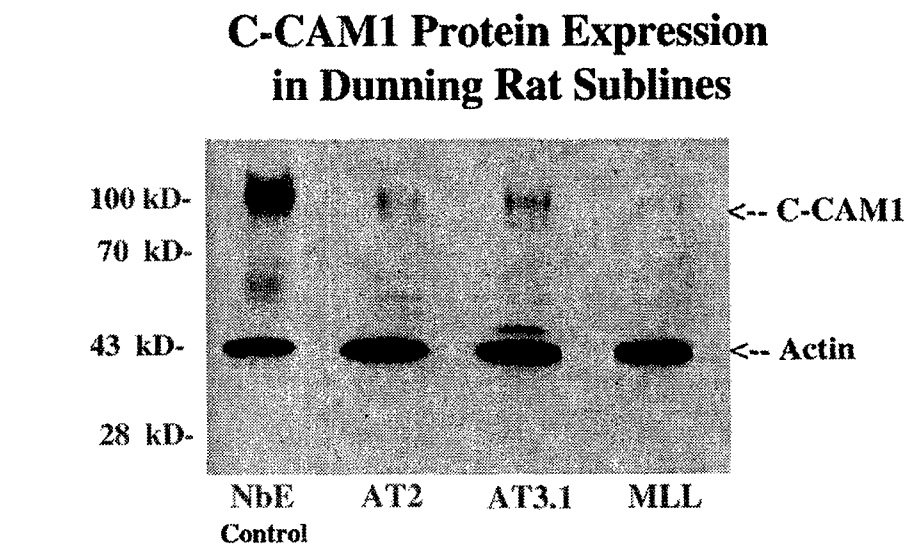
Fig. 2. Development of the Dunning tumor sublines.

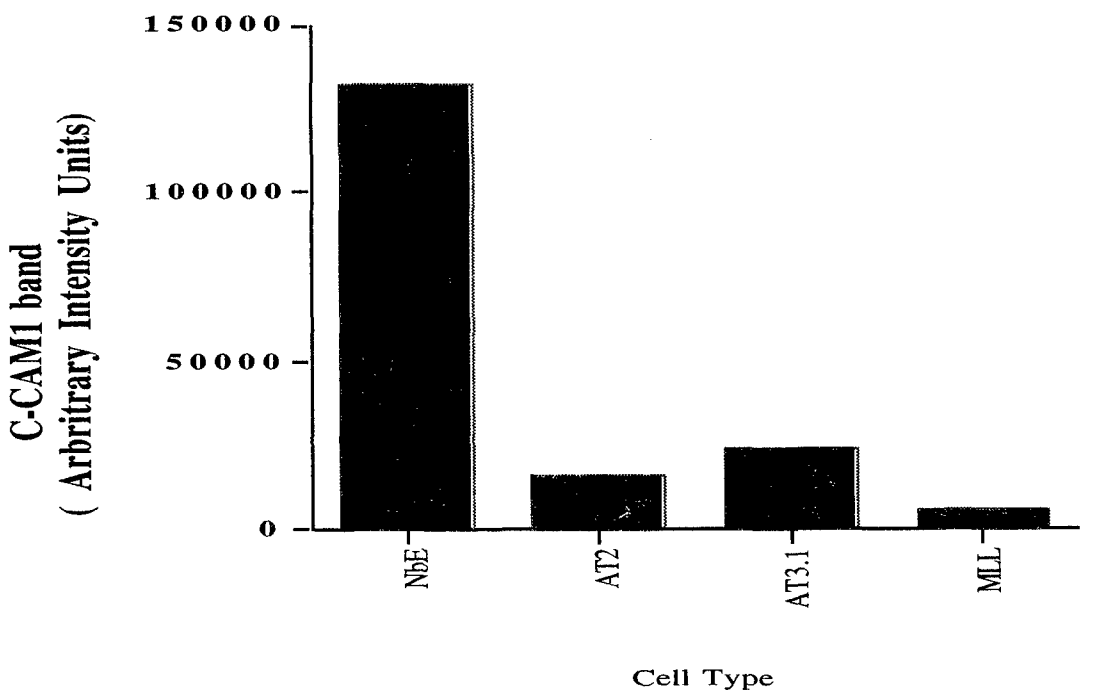


B.1.3.6. Western immunoblot analysis of C-CAM gene expression in the Dunning series prostate cancer cell lines

The levels of C-CAM protein expression in these cell lines were examined by western immunoblot analysis. A normal prostate cell line NbE, derived from ventral prostate of Noble rat (3), was used as a control. As shown in Fig. 3, distinct changes occurred in C-CAM expression in the Dunning prostate cancer cell lines. A significant decrease in C-CAM protein levels occurred at the transition from normal to carcinoma, i.e. Dunning 3327, followed with further reduction in C-CAM protein level in the rapid growing tumors, i.e. AT-3 and Mat-LyLu. In Mat-LyLu cell line, C-CAM expression level is about 5% as compared to that of normal control cell line. Due to this low C-CAM level, we chose to use Mat-LyLu cell line for further studies.

Fig. 3. Western immunoblot analysis of C-CAM expression in Dunning rat prostate cancer cell lines.



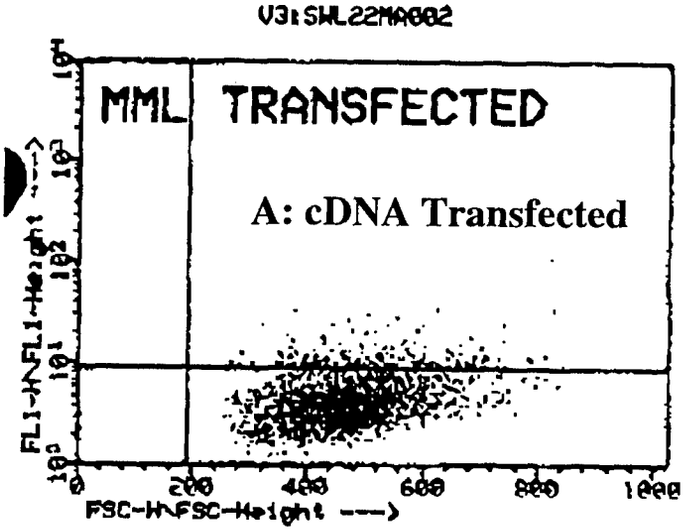


B.1.3.7. Transfection of human prostate cDNA library in mammalian expression vector into Mat-LyLu and FACS analysis

To screen for C-CAM activation factors present in normal prostate, a human prostate cDNA library in mammalian expression vectors was constructed and used to transfect Mat-LyLu cells. Three million Mat-LyLu cells were transfected with 15 ug of expression vector using lipofectin (Gibco/BRL) according to manufacturer's instruction. At 2 days post-

transfection, these cells were trypsinized from the plates and incubated with polyclonal anti-C-CAM antibodies followed with FITC-conjugated secondary antibody. The top 10% fluorescence positive cells, which were considered C-CAM positive, were separated from total cell populations by FACS. Fig. 4A shows the FACS profile and the population of cells sorted. Fig. 4B shows the fluorescence profile of the selected cells. It is obvious from the Fig. 4B that the selected cells indeed exhibited higher fluorescence as compared to that of total cell population.

Fig. 4. FACS sorting of Mat-LyLu cells transfected with prostate cDNA library. (A) FACS profile of the transfected cell population. (B) FACS profile of the cells selected.



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Selected Preference: Arit
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2 UR	679	9.07	←
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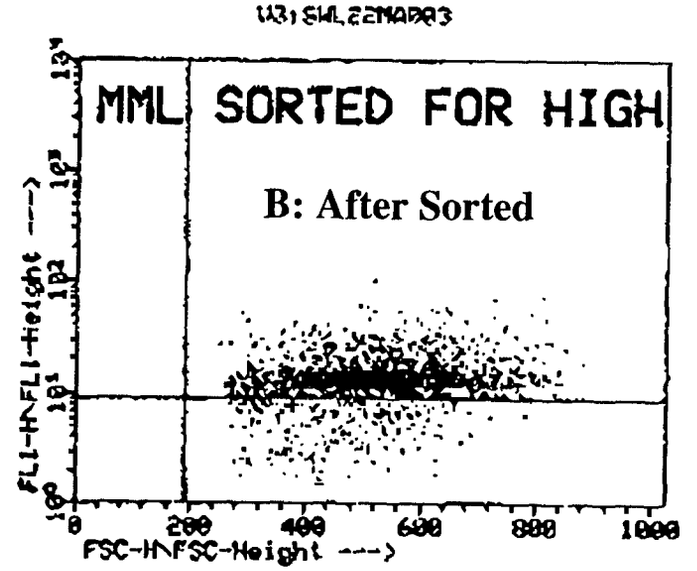
4 LR	6794	90.93	



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Quad	Events	% Gated	%
1 UL	0	0.00	
2 UR	5302	85.85	←
3 LL	0	0.00	
4 LR	874	14.15	

B.1.3.8. Isolation of plasmid DNA from C-CAM positive cells

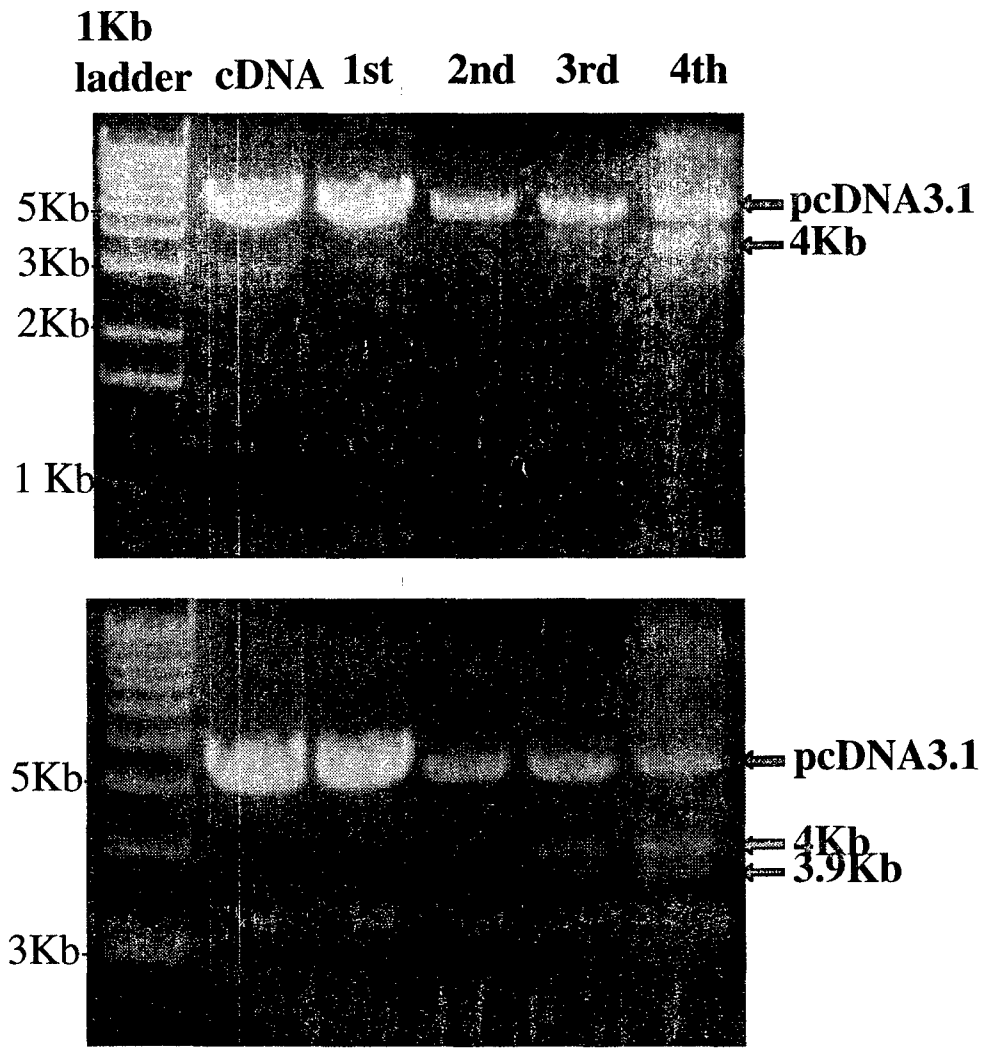
The selected cells were concentrated by centrifugation and the DNA were isolated with phenol/chloroform extraction followed with ethanol precipitation. Plasmid DNA, which contained b-lactamase gene, were ampicilin resistant and were retrieved from the total DNA by electroporation of total DNA into E. coli. Transformed bacteria were selected in ampicilin-containing agar plates. Fifty thousand ampicilin resistance colonies were recovered from the first round of selection. These bacteria colonies were combined and their plasmid DNA (first round DNA) were prepared using a Maxi-prep kit from Qiagen.

The first round DNA (15 ug) were used to transfect seven millions of Mat-LyLu cells. The highest 4.5% C-CAM positive cells were FACS sorted and the plasmid DNA (second round DNA) were retrieved as described above. The same procedures were repeated two more times and the resulting DNA were analyzed by restriction enzyme digestion.

B.1.3.9. Results from 4 cycles of FACS selection

Restriction digestion of plasmids after four-cycle sorting is shown in Fig. 5. The original cDNA library contained a series of DNA inserts with various sizes and appeared on the gel as a smear pattern. Enrichment of certain insert sizes was observed following four cycles of selection (Fig. 5). The plasmids from the fourth selection will be transformed into bacteria and plasmid from single bacteria colony will be isolated. These plasmid DNA will be transfected into Mat-LyLu to confirm their ability to activate C-CAM gene promoter. The plasmids which give consistent activation of C-CAM expression in Mat-LyLu cells will be further analyzed. The DNA sequences of these plasmid inserts will be determined by DNA sequencing analysis.

Fig. 5. Restriction digest profile of the plasmids isolated from cells selected from FACS sorting. The lower panel is the longer electrophoresis run of first panel.



B.1.3.10. Discussion

Our effort in the search for activator/co-activators that regulate C-CAM gene expression during tumorigenesis has yielded significant insights. We found that promoters of limited sizes might not contain regions responsible for tumor-specific regulation of C-CAM expression. This is illustrated by our first approach using a C-CAM promoter-driven GFP construct. Although C-CAM is not expressed in PC-3 cells, the 1609 bp C-CAM promoter was able to activate GFP expression in PC-3 cells. This observation suggests that either the 1609 bp promoter region does not contain any tumor-regulated promoter element or the 1609 bp promoter fragment is not sufficient to elicit the tumor-specific regulatory event. As a result, a longer promoter will be needed to address this question.

Alternatively, the study should be performed in a tumor cell line in which the level of C-CAM was down-regulated. We have found that Mat-LyLu prostate cancer cell line is suitable for our purpose of research. We have obtained some interesting results using this cell lines and will continue this line of investigation.

B.2. Studies on androgen regulation of C-CAM gene expression--(also see manuscript attached)

B.2.1. Rationale

Because C-CAM is an epithelial cell adhesion molecule and androgen is known to promote differentiation of prostatic epithelia, it is likely that C-CAM expression in prostate is regulated by androgen. In our previous studies using castration-induced prostate involution together with administration of androgen or antiandrogen, we found that expression of C-CAM in rat ventral prostatic epithelia was repressed by androgen (4, 5). A similar regulatory pattern was observed in seminal vesicle, but not in other organs (e.g. liver and kidney). These observations suggest that regulation of C-CAM expression by androgen is tissue-specific. However, it is not clear whether androgen regulation of C-CAM expression in rat ventral prostate is due to a direct effect on C-CAM gene expression or an indirect effect mediated by stromal-epithelial interaction. Therefore, we investigate whether androgen receptor has a direct or indirect effect on C-CAM promoter.

B.2.2. Experimental Plan

The effect of androgen and androgen receptor on C-CAM gene expression was studied by co-transfecting a reporter plasmid containing C-CAM promoter sequence and an expression plasmid containing androgen receptor. Mutation analysis was performed to locate the androgen response element located in the C-CAM promoter.

B.2.3. Results

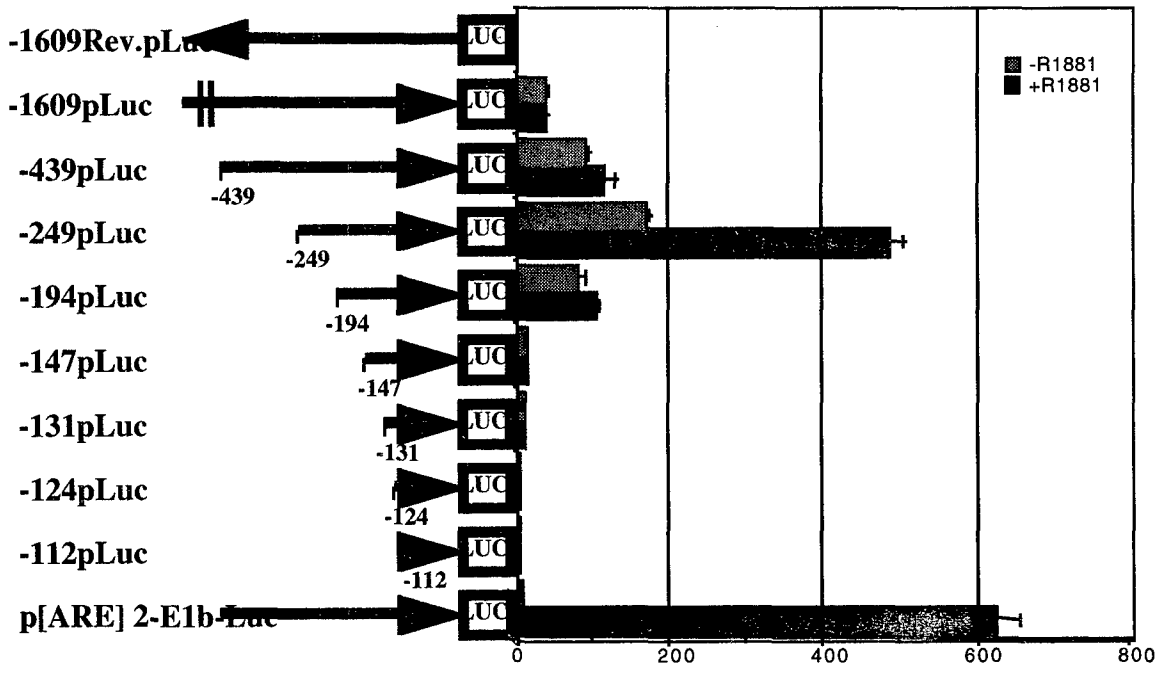
B.2.3.1. Localization of androgen-responsive region in C-CAM1 promoter

C-CAM promoters with differing length, constructed by 5' deletion, were cloned in front of luciferase gene in the reporter plasmid. Each of these plasmids, together with the androgen receptor expression vector pAR0, was transiently transfected into HeLa cells. The 1609 bp C-CAM promoter mediated a 106-fold increase in reporter gene (luciferase) expression without addition of androgen analogue R1881 (Fig. 6). Deletion of C-CAM promoter up to bp -194 did not have any significant effect on its

ability to induce luciferase expression, while deletion up to bp -147 markedly reduced the promoter activity (Fig. 6). This result suggests that a minimal promoter is located within the first 194 bp 5' from C-CAM's translation start site.

We next investigate whether there is any androgen responsive sequence in the C-CAM 5' promoter region. As shown in Fig. 8, the plasmid containing C-CAM promoter region from -249 bp to -21 bp exhibited a two-fold increase in luciferase activity upon addition of androgen analogue R1881. Plasmid containing the 439 bp segment proximal to the translation start site also gave a moderate hormone response (1.5 fold). In contrast, no hormone response was observed with plasmids containing the entire 1609 bp or the 194 bp segment proximal to the translation start site. These observations suggest that the region between -249 and -194 bp in C-CAM promoter may contain an androgen-regulated sequence.

Fig. 6. Regulation of the C-CAM expression by androgen. A series of reporter plasmids containing C-CAM promoter fragments with different 5' deletions were co-transfected with wild-type androgen receptor plasmid (pAR0) into HeLa cells. Twenty hours post-transfection, cells were incubated with (+) or without (-) 1 nM R1881. Luciferase activities of these cell lysates were determined and reported as averages ± S.D. in relative light units from triplicate transfections.

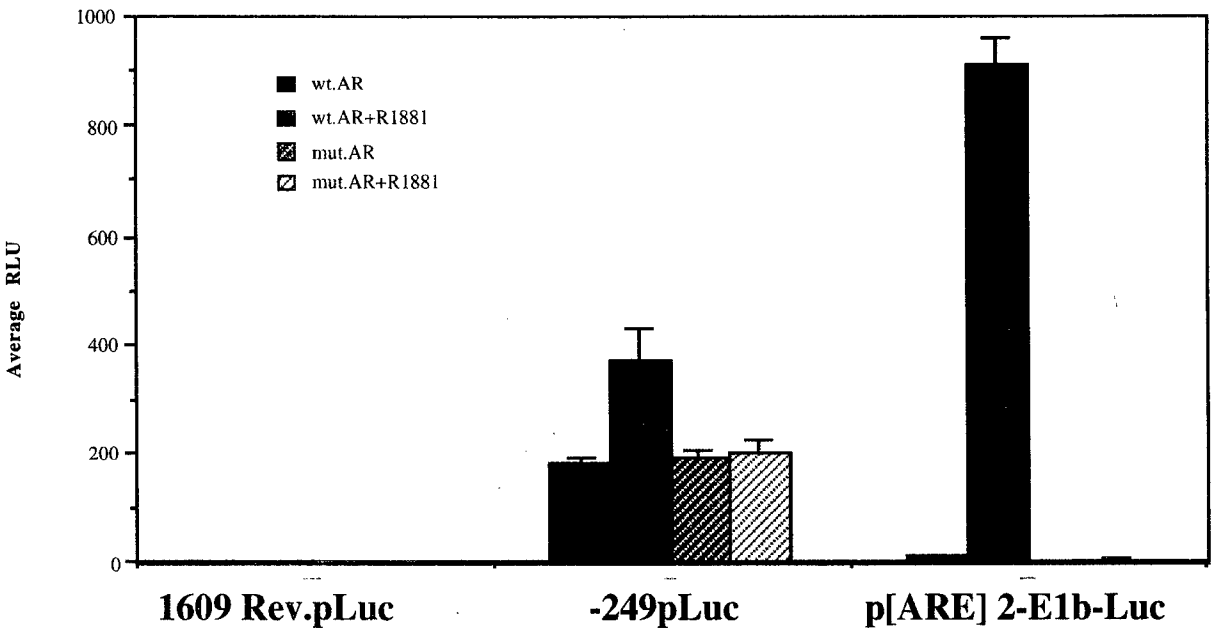


***In Vitro* Study**

B.2.3.2. Test the effect of mutant AR on C-CAM promoter activity

AR is a 100 kDa protein containing a DNA binding domain in its N-terminal region and a transcription activation domain in its C-terminal region. To test whether activation of C-CAM promoter by androgen is due to direct interaction between AR and C-CAM promoter, we have investigated the effect of mutant AR (AR64), which has a mutated (defective) DNA-binding domain, on C-CAM promoter activity. In contrast to the wild-type AR, this mutant AR (AR64), when co-transfected with p-249Luc into HeLa cells, did not show any detectable hormone induction (Fig. 7). This result suggests that activation of C-CAM promoter by wild-type AR is likely mediated by direct binding of AR to C-CAM promoter.

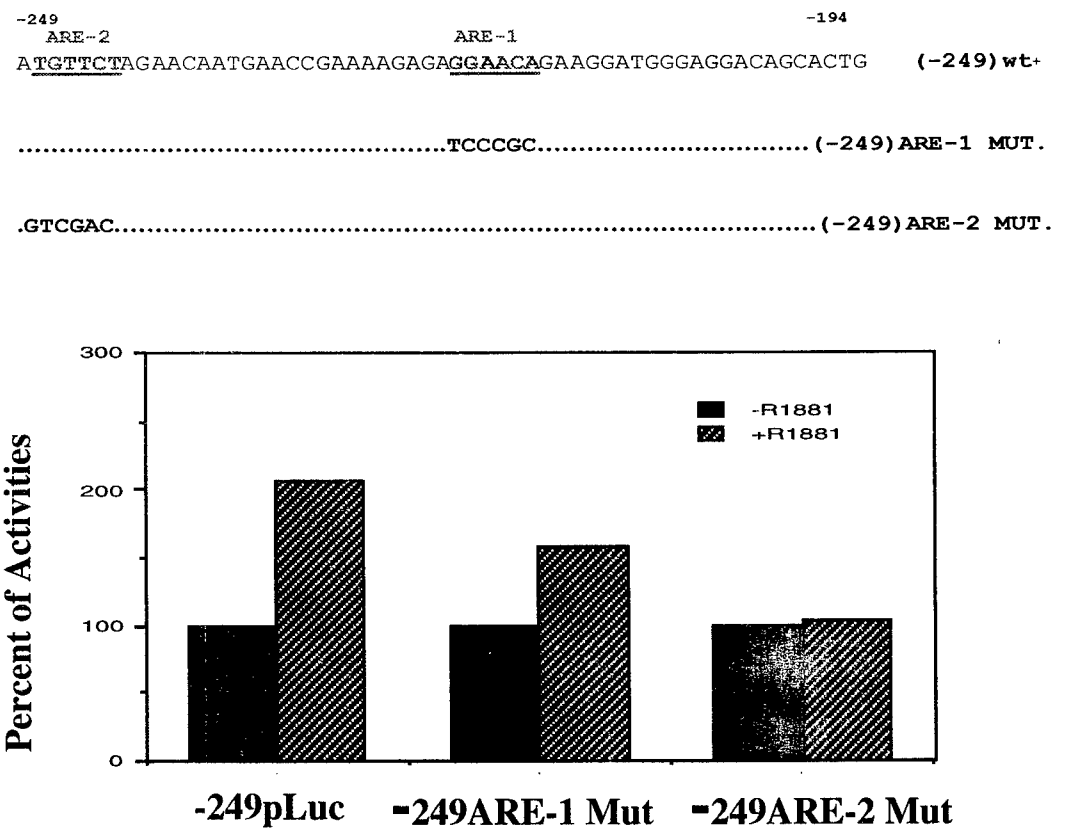
Fig. 7. Effect of AR mutant on its ability to activate C-CAM promoter. Cells were transfected with the -249Luc C-CAM promoter together with wild-type AR (pAR0) or mutant AR (pAR64) plasmid, respectively. Activities are presented as averages ± SD of triplicate transfections.



B.2.3.3. Identification of AR-interacting sites in C-CAM1 promoter

The consensus DNA-binding site for androgen receptor is GGA/TACANNNTGTTCT (6). Two potential androgen-responsive element (ARE) half sites, GGAACA and TGTTCT, located at bp -215 and -248 in C-CAM 5' promoter region, respectively, were found (Fig. 8). These half sites are found in the same region, i.e., between not -249 and -197 in C-CAM 5' promoter, where the androgen-regulated sequence was identified. These two potential ARE half sites (ARE-1 and ARE-2) were mutated to see if they are indeed involved in androgen regulation. Mutation of the first site from GGAACA to TCCCGC (ARE mutant #1) did not have any detectable effect on C-CAM promoter's response to R1881, suggesting that this sequence is probably not involved in androgen regulation of C-CAM. On the other hand, mutation of the second site from TGTTCT to GTCGAC abolished the androgen-stimulated response. These results suggest that the sequence between no -248 and -243 (ARE-2) is critical for androgen regulation of C-CAM, whereas that between not -215 and -210 (ARE#1) is not.

Fig. 8. Mutational analysis of the two putative androgen-responsive elements. Cells were transfected with reporter plasmids containing the -249 bp promoters whose putative ARE-1 or ARE-2 sequence was mutated. Activities are presented as percent of that of the corresponding plasmid containing no mutation and without R1881 treatment.



B.2.3.4. Discussion

We show that AR up-regulates C-CAM transcription in a ligand-dependent manner and this up-regulation requires a short (-249) promoter. In addition, AR, through its DNA binding domain, directly interacts with C-CAM promoter. These observations suggest that AR regulation of C-CAM expression is, at least in part, mediated by a direct mechanism. This study also establishes that androgen receptor is one of the transcriptional regulator of C-CAM gene.

B.3. Studies performed under Task 2--Examine the involvement of AP-2 in C-CAM gene expression

B.3.1. Rationale

The C-CAM promoter does not have TATA or CAAT box but has potential binding sites for known basal and regulatory transcriptional factors. The minimal promoter has been shown to localize in the 194 bp region upstream of the translational start site in normal prostate cell line NbE. In this region, the transcriptional factors that are known to bind to DNA elements include SP-1 and AP-2. Since SP-1 is a factor for basal transcription of many genes, it is unlikely to be involved in the tumor-specific regulation of C-CAM expression. Thus, AP-2 is the only known factor that is potentially involved in the down regulation of C-CAM in tumorigenesis.

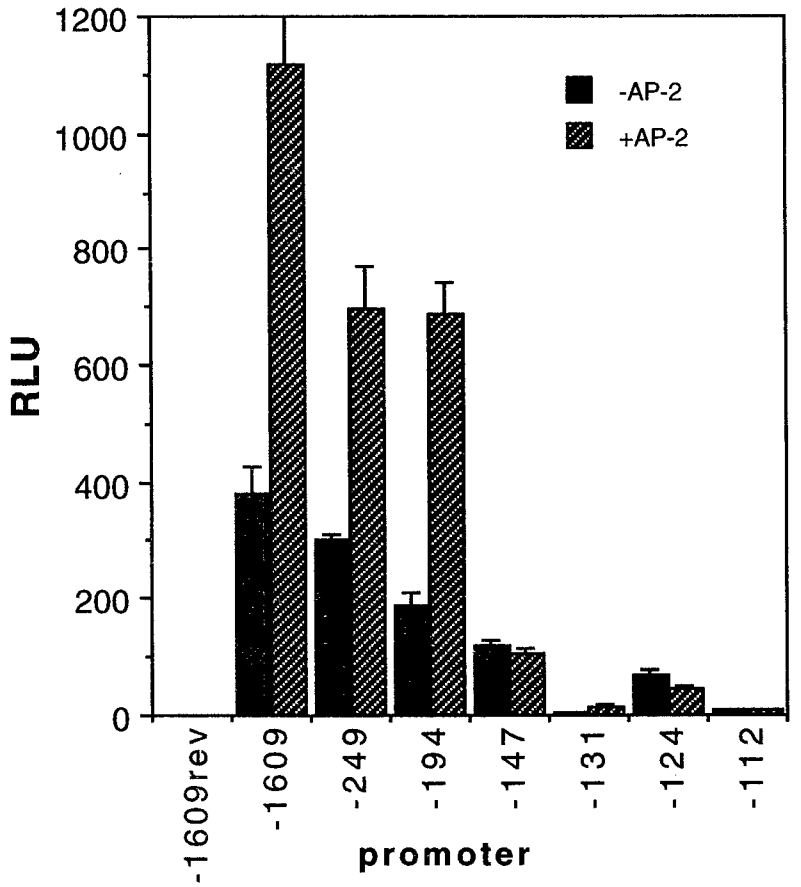
B.3.2. Experimental Plan

We examine whether AP-2 has any effect on C-CAM promoter activity by co-transfection of C-CAM promoter plasmid constructs containing various length of C-CAM promoter with expression plasmid containing AP-2 cDNA.

B.3.3. Results

C-CAM promoters with differing length, constructed by 5' deletion, were cloned in front of luciferase gene in the reporter plasmid. Each of these plasmids was transiently co-transfected with or without the mammalian expression plasmid containing AP-2 gene into HeLa cells. Addition of AP-2 resulted in a 2.5-fold increase in reporter gene (luciferase) expression when the reporter gene was driven by C-CAM promoter containing 1609, 249, or 194 bp (Fig. 9). Deletion of C-CAM promoter down to 147 bp abolished the AP-2 effect on C-CAM promoter (Fig. 9). This result suggests that AP-2 is one of the transcriptional activator of C-CAM promoter and the AP-2 responsive element is located between 194 bp to 147 bp region 5' from C-CAM's translation start site. This observation is consistent with the AP-2 binding site predicted from sequence analysis.

Fig. 9. Regulation of the C-CAM expression by AP-2. A series of reporter plasmids containing C-CAM promoter fragments with different 5' deletions were co-transfected with mammalian expression vector containing AP-2 gene into HeLa cells. Luciferase activities of these cell lysates were determined and reported as averages ± S.D. in relative light units from triplicate transfections.



B.3.4. Discussion

This result suggests that AP-2 is one of the transcriptional activator of C-CAM promoter and thus it likely participates in the regulation of C-CAM expression during tumorigenesis. To further examine the role of AP-2 in C-CAM gene expression in carcinogenesis, we will study whether expression of AP-2 is altered in prostate cancer cells. We will perform Northern analysis on RNA samples isolated from prostate cancer cells and normal prostate tissues. If AP-2 shows a similar pattern of down-regulation as that of C-CAM, it is very likely that it plays a role in the reduced C-CAM expression during carcinogenesis. The full-length cDNA coding for AP-2 will then be transfected into prostate cancer cells. The ability of AP-2 to activate C-CAM gene expression in prostate cancer cells will be tested by transient transfection. The functional consequence of AP-2 expression will be examined by selecting the transfectants and test whether these AP-2 expressing prostate cancer cells have lower *in vivo* tumorigenicity in nude mice.

(C) Key research accomplishments: Bulleted list of key accomplishments from this research.

- The effort in the search for activator/co-activators that regulate C-CAM gene expression during tumorigenesis has yielded significant insights. We found that Mat-LyLu prostate cancer cell line is suitable for our research purpose and have obtained some interesting results using this cell line.

- We show that AR up-regulates C-CAM transcription in a ligand-dependent manner. In addition, AR, through its DNA binding domain, directly interacts with C-CAM promoter. These observations suggest that AR regulation of C-CAM expression is, at least in part, mediated by a direct mechanism.

- We showed that AP-2 is one of the transcriptional activators of C-CAM promoter and thus AP-2 likely participates in the regulation of C-CAM expression during tumorigenesis.

(D) Reportable outcomes

1 manuscript in preparation for publication

Phan, D., Jenster, G., Luo, W., Sui, X., Najjar, S., and Lin, S.-H.: Transcriptional regulation of C-CAM1 gene by androgen receptor.

1 abstract to be presented at 10th International Workshop of CEA family genes (Sept. 2-5, 1999)

Phan, D., Jenster, G., Luo, W., Sui, X., Najjar, S., and Lin, S.-H.: Transcriptional regulation of C-CAM1 gene by androgen receptor.

(E) Conclusions

We propose to unravel mechanisms that regulate C-CAM gene expression in prostate carcinogenesis. Identification of earliest genetic changes in tumorigenesis will likely yield targets for prevention and early detection. In addition, therapeutic approaches targeting these early changes might lead to more effective cancer treatment. We have identified transcriptional factors, including AP-2 and androgen receptor, that are involved in the regulation of C-CAM gene expression. In addition, we have developed an *in vivo* functional screening method to identify new

transcriptional factors that regulate C-CAM gene expression during prostate carcinogenesis. Results from this study will allow us to design new therapy strategies to alter tumor progression or to implement early detection and prevention strategies.

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(G) Appendices

one manuscript entitled: Transcriptional regulation of C-CAM1 gene by androgen receptor. by Phan, D., Jenster, G., Luo, W., Sui, X., Najjar, S., and Lin, S.-H.

Transcriptional Regulation of C-CAM1 Gene by Androgen Receptor

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Running Title: Androgen regulation of C-CAM1 gene transcription

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abbreviations used: C-CAM, cell-cell adhesion molecule; CEA, carcinoembryonic antigen; Ig, immunoglobulin; BGP, biliary glycoprotein; PCR, polymerase chain reaction.

The nucleotide sequences used in this paper are in the GenBank/EMBL Data Bank with accession numbers U27207 for 5'-flanking region of rat C-CAM/pp120 gene.

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Abstract

Loss of C-CAM expression is an early event in prostate cancer progression. We previously showed that expression of C-CAM in prostate cancer cells could suppress their tumorigenicity *in vivo*. These observations suggest that C-CAM functions as a tumor suppressor in prostate cancer. Because androgen receptor (AR) is an important transcription factor that regulates prostate function, we investigted the effects of AR on C-CAM transcription. Regulation of C-CAM expression by the AR was studied in HeLa cells by co-transfecting an AR expression vector with different reporter plasmids under the control of various fragments of the C-CAM promoter. We showed that the AR up-regulated the transcription from specific fragments of C-CAM promoter activity in a ligand-dependent manner and that the regulatory element resides within a relatively short (-249 to -194) stretch in the 5' flanking region of the C-CAM gene. This androgen-regulation is very likely through direct AR-promoter binding since mutant AR defective in DNA binding failed to up-regulate reporter gene expression. Two androgen-response elements (ARE) with homology to the androgen response element (ARE) consensus sequence were identified in the -249 to -194 region. Mutation of one of these putative AREs abolished the ligand-dependent response, suggesting that the AR binds to this specific sequence, thereby directly regulates C-CAM expression.

INTRODUCTION

C-CAM is a cell adhesion molecule and a member of the immunoglobulin supergene family (1, 2). C-CAM is mainly expressed in epithelial cells of many different tissues including the prostate (3). Loss of C-CAM is an early event in prostate cancer progression (4, 5), suggesting that C-CAM may play an important role in prostate tumorigenesis. Consistent with this hypothesis, expression of C-CAM in prostate cancer cells can suppress their tumorigenicity *in vivo* (6). These observations suggest that C-CAM functions as tumor suppressor in prostate cancer.

The prostate is an androgen-dependent organ; androgen is the major regulator of prostate development, growth and secretory function. Induction of prostate involution and cancer regression by androgen ablation is one of the most effective treatments for late stage prostate cancer. Since C-CAM is a tumor suppressor of prostate cancer, it is important to know whether expression of C-CAM in prostate is regulated by androgen. Using castration-induced prostate involution followed by administration of androgen or anti-androgen, we found that C-CAM expression in the rat ventral prostate was negatively regulated by androgens (7). A similar regulatory pattern was observed in the seminal vesicle but not in the dorsal prostate or other organs (e.g., liver and kidney). These observations suggest that regulation of C-CAM expression by androgens is tissue-specific.

Alternative splicing of the C-CAM gene produces two-isoforms (L-form and S-form) with different lengths of their cytoplasmic domains (8). Only the L-form C-CAM, which has a cytoplasmic domain of 71 amino acids, has

tumor suppressive activity and the S-form C-CAM, with only 10 intracellular amino acids, does not (9). Immunohistochemical studies using isoform-specific antipeptide antibodies showed that both C-CAM isoforms were undetectable in normal ventral prostate. However, androgen depletion by castration, induced a marked increase in both isoforms in the ventral prostate and both L- and S- variants were found to co-express in the same cells (10). These studies establish that expression of C-CAM gene in the ventral prostate is regulated by androgens and that both C-CAM isoforms are regulated by androgens in a concerted fashion.

The rat C-CAM promoter belongs to the GC-rich class of TATA-less promoters (11). Deletions and substitution analyses revealed that the three proximal Sp1 binding sites are essential for basal transcription of the C-CAM gene. In addition, Najjar et al. (11) have shown that C-CAM promoter activity was stimulated 2-3 fold by insulin, dexamethasone, and cAMP. However, the effect of androgens on C-CAM promoter activity had not been examined.

Previous studies have revealed that the stromal component of the prostate has a strong influence on both normal prostate development and prostate cancer progression (12-14). It therefore, is not clear whether androgen regulation of C-CAM expression in rat ventral prostate is due to a direct effect on C-CAM gene expression or an indirect effect mediated by stromal-epithelial interactions. In this study, we examined whether the AR has a direct or indirect effect on the C-CAM promoter.

MATERIALS AND METHODS

Plasmid constructions

The 5'-flanking region of rat C-CAM gene was cloned as previously described [Najjar, 1996 #171]. Using PCR, 5' deletion products (-1609bp, -439bp, -249bp, -194bp, -147bp, -131bp, -124bp, -112bp) of the C-CAM gene were synthesized and subcloned at the XhoI and HindIII sites of pGL3-BASIC plasmid [Najjar, 1996 #171].

Mutants p-249(ARE-1) and p-249(ARE-2) were generated by site-directed mutagenesis of the p-249Luc vector using PCR. Oligo #305 (forvard primer) (AAGCTTTTCTCTTGGGGAAGA) and oligo #306 (reverse primer)(CTCGAGATGTTCTAGAACAATGAACCGAAAAGAGATCCCGCGAAGGATG GGAGGACA) were used as primers to introduce substitutions in the ARE-1 region, while oligo #307 (forvard primer) (GCTAGCCCGGGCTCGAGAGTCGACAGAACAATGAACCG) and #306 were used to introduce substitutions in the ARE-2 region. After these PCR products were sequenced to confirm the mutations, they were subcloned at the XhoI and HindIII sites of pGL3- BASIC plamid (Promega). The reporter plasmid harboring two androgen/progesterone/glucocorticoid response elements and a TATA-box driving the luciferase gene ([ARE]2-E1b-luc) have been described previously (15).

The human AR cDNA expression vector (pAR0) was constructed with the SV40 early promoter and the rabbit ß-globin poly-adenylation signal as previously described (16). AR mutant pAR64, whose first zinc finger in the

AR is disrupted by the replacement of two cysteines with serine and phenylalanine, was constructed as described in Jenster et al. (17).

Cell Culture and Transfection of HeLa cells

HeLa cells (human epithelial cervix carcinoma (American Type Culture Collection)) were maintained in minimal essential medium supplemented with 10% fetal calf serum. These cells (50,000) were plated in a 12-well plate with 10% charcoal-stripped fetal calf serum (CC-FCS) 24h before transfection. Cells were transfected with 0.3 ug of luciferase reporter plasmid containing C-CAM promoter fragment, and 0.3 ug of receptor plasmid containing either wild type (pAR0) or mutant androgen receptor (pAR64) per well using Lipofectin (Life Technologies, Inc.) according to the manufacturer's guidelines. Twenty four hours post-transfection, cells were washed and fed with medium containing stripped serum with or without R1881 (17a-methyltrienolone) (NEN Life Science Products.) and the incubation was continued for an additional 24 h. Cells were lysed in 200 ul lysis buffer (Promega) and luciferase activity was measured using a luciferase assay system (Promega). Experiments were performed in triplicate.

RESULTS

Localization of an androgen-responsive region in the C-CAM1 promoter

C-CAM promoters with different lengths, constructed by 5' deletion, were cloned in front of the luciferase gene in the reporter plasmid. Each of these plasmids were transiently co-transfected with the androgen receptor expression vector pAR0 into HeLa cells (16). In the absence of the androgen

analogue R1881, the 1609 basepair (bp) C-CAM promotor mediated a 106-fold increase in reporter gene expression as compared to the reverse oriented C-CAM promoter fragment (Fig. 1). Deletion of the C-CAM promotor up to bp -194 did not have any significant effect on its ability to induce luciferase expression, while deletion up to bp -147 markedly reduced the promoter activity. This result suggests that a minimal promoter is located within the first 194 bp 5' from C-CAM's translation start site. This result is consistent with a previous report (11). We next investigated whether there is any androgen responsive sequence in C-CAM promoter. As shown in Figure 1, the plasmid containing the C-CAM promoter region from bp –249 to bp -21 exhibited a two-fold increase in luciferase activity upon addition of androgen analogue R1881. The plasmid containing the 439 bp segment proximal to the translation start site also gave a slight hormone response (1.5 fold). In contrast, no hormone response was observed with plasmids containing the entire 1609 bp or the 194 bp segment proximal to the translation start site. These observations suggest that the region between bp -249 and -197 in C-CAM gene may contain an androgen-regulated sequence.

Effect of mutant AR on C-CAM promoter activity

The AR is a 110-112 kDa protein containing transcriptional activation domains in its N-terminal region, a centrally located DNA binding domain, and the ligand binding domain at its C-terminus. To test whether activation of C-CAM promoter by androgen is due to direct interaction between AR and C-CAM promoter, we investigated the effect of a mutant AR (AR64), which has a mutated DNA-binding domain resulting in defective DNA binding (17), on C-CAM promoter activity. In contrast to the wild-type AR, the mutant

AR (AR64), when co-transfected with p-249Luc into HeLa cells, did not show any detectable hormone induction (Fig. 2). This result strongly suggests that activation of the C-CAM promoter by the wild-type AR is mediated by direct binding of the AR to the C-CAM promoter.

Identification of AR-interacting sites

A consensus DNA-binding site for the AR has been identified using a PCR based random primer gel shift technique (18). The consensus sequence GGA/TACANNNTGTTCT (18). Two potential AREs with one perfect half site, GGAACA and TGTTCT are located at bp -215 and -248 in the C-CAM promoter and could be responsible for androgen-induction of the -249 reporter activity (Fig. 3). The two perfect ARE half sites (ARE-1 and ARE-2) were mutated to see if they are indeed involved in androgen regulation. Mutation of the first site from GGAACA to TCCCGC (ARE mutant #1) did not affect C-CAM promoter's response to R1881, suggesting that this sequence is probably not involved in androgen regulation of C-CAM (Fig. 3). On the other hand, mutation of the second site from TGTTCT to GTCGAC abolished the androgen-stimulated response (Fig. 3). These results suggest that the sequence between bp -248 and -243 (ARE-2) is critical for androgen regulation of C-CAM, whereas that between nt -215 and -210 (ARE-1) is not.

DISCUSSION

We show here that the AR up-regulates a fragment of the C-CAM promoter in a ligand-dependent manner and that this up-regulation requires an intact AR DNA binding domain. These observations suggest that AR regulation of C-CAM expression is mediated by a direct mechanism.

Androgen receptors regulate gene transcription through a multistep process that requires direct binding of AR to specific DNA sequences known as androgen-responsive elements (AREs) (Chang et al., 1995). Using a DNA-binding site selection assay, Roche et al. (18) have determined a consensus AR DNA-binding site: 5'-GGA/TACANNNTGTTCT-3'. This consensus sequence contains two half sites with three intervening basepairs. However, this topology is obviously not strictly conserved. For example, a variety of AR binding sequences with significant deviations from this "consensus" sequence have been reported (19, 20). For example, the androgen-responsive elements in rat probasin 5' UTR were determined to be 5'-ATCTTGTTCTTAGT (ARE-1), located between nt -236 and -223, and 5'-GTAAAGTACTCCAAGAACCTATTT (ARE-2), located between nt -140 and -117 (Rennie et al., 1993). Only one of these sequences, i.e., ARE-1, contains a half site of the canonical "consensus" sequence and these two AREs are separated by 82 nucleotides. Similarly, the two AREs that are required for cell-specific up-regulation of androgen receptor transcription contain only a half site in one of the AREs and are separated by 182 nucleotides (20).

Studies by Rennie et al. (19) have shown that androgen regulation of probasin gene involves two cis-acting elements, ARE-1 and ARE-2, with which AR interacts directly. When both ARE-1 and ARE-2 are present, probasin promotor can be activated 30- to 40-fold by androgen. However, no androgen activation of probasin promoter was observed when ARE-2 was absent, while a less than 2-fold androgen induction of probasin promoter activity was obtained when ARE-1 was absent. In our study, only a two-fold stimulation by androgen was observed which can be attributed to only one ARE half site within the androgen-responsive region. This property is

similar to that of probasin promoter in that one ligand binding site can only provide weak transcriptional activation.

Using castration-induced prostate involution followed by administration of androgen, we have previously shown that C-CAM expression in rat ventral prostate epithelia is negatively regulated by androgen (7). In the present study, we found that androgen up-regulated C-CAM expression in a ligand-dependent manner. However, up-regulation of C-CAM expression by androgen was observed only with a relatively short promotor (bp -294 to -197 in C-CAM promoter), whereas the longer promoter, i.e. 1609 bp, failed to show any androgen response. This observation suggests that the *in vivo* regulation of C-CAM expression by AR may result from complex androgen-regulated events.

Interestingly, although androgen receptor is expressed in all prostate lobes, C-CAM expression is regulated by androgen only in ventral prostate but not in dorsal/lateral prostate [Hsieh, 1994 #1; Makarovskiy, 1999 #422]. Such lobular-specific regulation lends further support to the hypothesis that androgen regulation of prostate gene expression involves factors other than androgen/androgen receptor. The interactions between prostatic epithelial and stromal components have been shown to contribute to androgen-dependent regulation of prostate growth and differentiation (13, 14). As a result, although direct interactions between AR and C-CAM promoter may contribute to androgen regulation of C-CAM expression, other factors including signals from stromal-epithelial interactions may also influence C-CAM expression *in vivo*.

FIGURE LEGEND

Fig. 1. Regulation of the C-CAM expression by androgen. A series of reporter plasmids containing C-CAM promoter fragments with different 5' deletions were co-transfected with wild-type androgen receptor plasmid (pAR0) into HeLa cells. Twenty hours post-transfection, cells were incubated with (+) or without (-) 1 nM R1881. Luciferase activities of these cell lysates were determined as described in "Materials and Methods" and reported as averages ± S.D. in relative light units from triplicate transfections.

Fig. 2. Effect of AR mutant on its ability to activate C-CAM promoter. Cells were transfected with the -249 C-CAM promoter together with wild-type AR (pAR0) or mutant AR (pAR64) plasmid, respectively. Activities are presented as averages ± S.D. of triplicate transfections.

Fig. 3. (A) Nucleotide sequence between nt -249 and +16 in rat C-CAM 5'-UTR. Nucleotides are numbered relative to +1 at the ATG translation initiation codon and are labeled as negative numbers to reflect their position as upstream (5') of the ATG site. Two putative androgen-responsive elements (ARE-1 and ARE-2) are underlined. (B) Mutational analysis of the two putative androgen-responsive elements. Cells were transfected with reporter plasmids containing the -249 bp promoters whose putative ARE-1 or ARE-2 sequence was mutated as described in "Materials and Methods". Activities are presented as percent of that of the corresponding plasmid containing no mutation and without R1881 treatment. Data are presented as means ± S.E. of three independent experiments.

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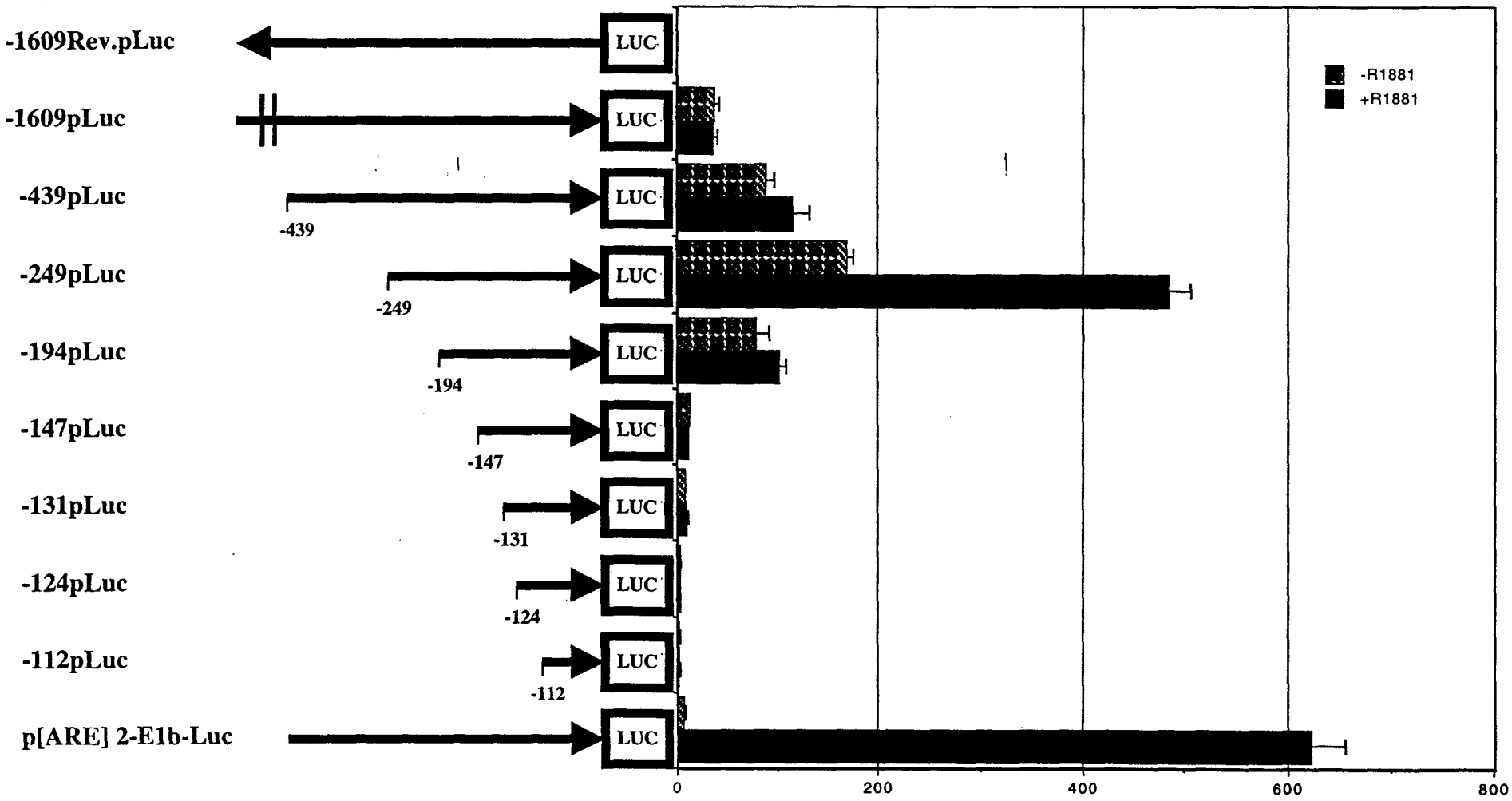
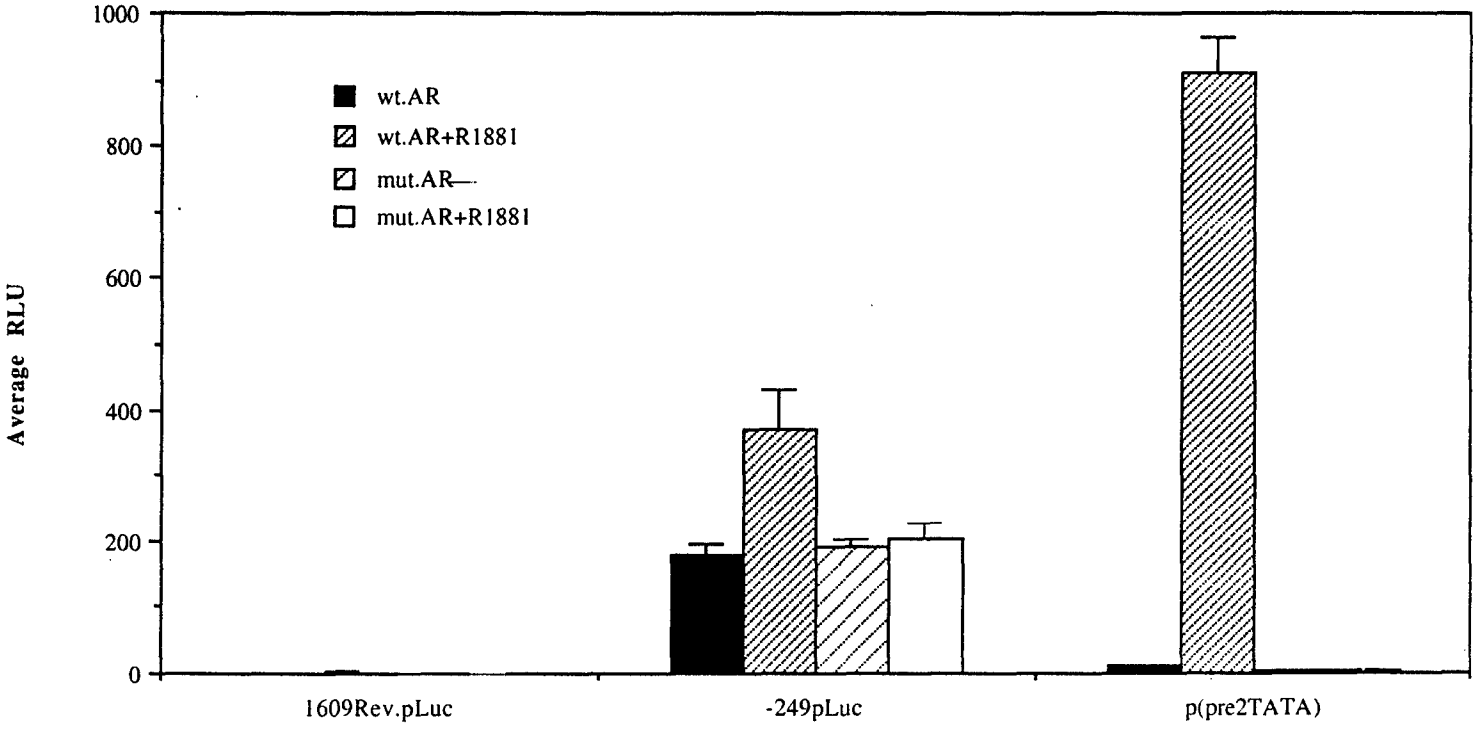
Localization of an Androgen-Responsive Region in the CEACAM1 Promoter



In Vitro Study

Direct Binding of AR to C-CAM1 Promoter Region



Partial C-CAM Promoter

actctaggct attggaaggg caggaattcc tgctgctgct gctgctggcc acaaggccag -285

-249 **ARE-2**

ggcacaggac acttctcagc cttgctgctc aaagt**atgtt ct**agaacaat gaaccgaaaa -225

ARE-1 -194

gaga**ggaaca** gaaggatggg aggacagcac tgagagaaca gcattgtcag agtcgcaggc -165

tccacccccc agccca*cgtg* *actctgg*gag gtgccctcct ctgggaggag actcgcttct -105

gctatgaaaa gcagggtggc taccagagca gccaggcttg gcaggcagcc gtgttggagc -45

caaatctctt ccccaagaga agaacctagc aggcagcaga gact***atg***gag ctagcctcgg +16

Mutation Analysis of the Two Putative Androgen-Responsive Elements

-249 -194

ARE-2 ARE-1

ATGTTCTAGAACAATGAACCGAAAAGAGAGGAACAGAAGGATGGGAGGACAGCACTG (-249)wt+

.....TCCCGC..... (-249)ARE-1 MUT.

.GTCGAC..... (-249)ARE-2 MUT.